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An improved method to determine serine palmitoyltransferase activity

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Abbreviated title: Improved method to determine SPT activity

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Footnotes:

The abbreviations used are: SPT, serine palmitoyltransferase; 3KDS, 3-ketodihydrosphingosine; OPA, ortho-phthalaldehyde; PyrDH, pyruvate dehydrogenase; SML, sucrose monolaurate; CerS, ceramide synthase; FB1, fumonisin B₁; LOD, limit of detection

Abstract

Serine palmitoyltransferase (SPT) catalyses the condensation of L-serine and palmitoyl-CoA, which is the rate-limiting step in the *de novo* synthesis of sphingolipids. SPT activity is commonly measured by monitoring the incorporation of radiolabeled L-serine into 3-ketodihydrosphingosine. In this article we introduce several adaptations of the established protocol to improve sensitivity, reproducibility and practicability of the assay. A significant improvement of this new protocol is the possibility to measure SPT activity in total cell lysate instead of microsomes. The assay is furthermore extended by the introduction of a non-radioactive, HPLC-based detection protocol. The suggested HPLC method offers several advantages, most importantly, a 20-fold lower detection limit compared to the radioactive assay and the possibility to use an internal standard to correct for variation in the extraction.

Supplementary keywords: sphingolipids, HPLC

Introduction

Serine palmitoyltransferase (SPT) [EC 2.3.1.50] catalyses the first, rate-limiting step in the *de novo* synthesis of sphingolipids – the condensation of palmitoyl-CoA and L-serine to 3-ketodihydrosphingosine (3KDS) (1, 2).

Sphingolipids are bioactive metabolites and modulate the activities of various enzymes, such as protein kinases, protein phosphatases and phospholipases in cells or cell-free systems (3). They are involved in many cellular events including proliferation, differentiation, senescence, apoptosis and inflammatory responses (4). Abnormalities in the function of SPT cause clinical disorders, such as hereditary sensory neuropathy type I. (5, 6). Recent studies also indicate that human plasma sphingolipid levels are an independent risk factor for coronary artery disease (7) and that lowering sphingolipid levels by pharmaceutical interventions can reduce the risk for atherogenesis in apolipoprotein E-knockout mice (8). Other data indicate that sphingolipids are involved in the pathological aggregation of amyloid- β fragments, which is a key process in the development of Alzheimer's disease (9).

To address these issues on a biochemical level a robust, sensitive and reproducible SPT activity assay would be desirable. SPT activity is usually determined by the method described by Merrill and Williams (10, 11), using [^3H]- or [^{14}C]-L-serine as a substrate, or variants thereof.

In this work we looked at various aspects of this method and suggest several adaptations for simplification and to improve assay precision and sensitivity. We furthermore provide a protocol for a standardised SPT activity assay to improve reproducibility and inter-assay comparability.

Experimental procedures

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-[U- ^{14}C]-serine was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). C₁₇- and C₂₀-sphingosine were from Avanti Polar Lipids (Alabaster, AL, USA).

Photometric assays

Thioesterase activity was measured photometrically by monitoring the reaction of free CoA with DTNB at 412 nm in a spectrophotometer (12). Concentrations were determined with a molar absorption

coefficient of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ for the 2-nitrobenzoate anion. The assay typically contained 50 μM palmitoyl-CoA, 100 μM DTNB and the corresponding amount of cells as stated in the text.

The influence of DTT on the release of free CoA from the substrate was determined enzymatically using pyruvate dehydrogenase (PyrDH). The assay contained 50 mM HEPES (pH 8), 50 μM palmitoyl-CoA, 1 mU PyrDH, 1 mM sodium pyruvate, 2.5 mM thiamine pyrophosphate, 2.5 mM MgCl_2 , 2.5 mM NAD^+ , 100 μM NADH, 0.5 mg/ml BSA, 50 mM sodium oxamate (to inhibit the lactate dehydrogenase) and the corresponding amount of DTT. NADH oxidation was monitored spectrophotometrically at a wavelength of 340 nm using an absorption coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. Background reactions were monitored in control samples without palmitoyl-CoA.

Photometric assays were usually performed in a 96-well polystyrene flat-bottom microtiter plate (Nunc, Roskilde, Denmark) and measured using a microplate spectrophotometer (PowerWave 340, BioTek, Winooski, VT, USA).

Sample preparation, SPT reaction and lipid extraction

Human Embryonic Kidney cells (HEK293) were grown in DMEM supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (100 $\mu\text{g/ml}$). Cultures were maintained at 37 °C in a 5% CO_2 atmosphere at 100% humidity. Cell membrane microsomes were prepared as follows: A HEK293 cell monolayer in a 10-cm dish was washed twice with 3 ml of PBS and suspended in 500 μl of 50 mM HEPES (pH 8) and 1 mM EDTA. The cell suspension was sonicated for 15 s at 50% power and 50% pulsation (Sonopuls HD 2070, Bandelin, Berlin, Germany) and centrifuged at 2'500g for 2 min. The supernatant was then centrifuged at 100'000g for 30 min and the pellet was resuspended in the desired buffer (usually 50 mM HEPES (pH 8) and 1 mM EDTA). Preparation of total cell lysate, SPT reaction and lipid extraction were performed as described in the appendix. Protein determinations were performed according to Bradford (13), using the protein assay kit from BioRad (Hercules, CA, USA).

For some experiments a prokaryotic, soluble form of SPT from *Sphingomonas paucimobilis* was used (14). The protein was expressed in *Escherichia. coli* (BL21 star (DE3)) using the pET expression system (pET21b, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were lysed in 50 mM HEPES (pH 8), 5 mM EDTA by sonification and stored at -20 °C in 50% glycerol.

Detection of radioactivity by liquid scintillation counting

Radioactivity was determined using Ultima Gold (PerkinElmer, Wellesley, MA, USA) liquid scintillation cocktail and a Packard Tri-Carb 1900TR (PerkinElmer) scintillation counter.

Product detection by high performance liquid chromatography (HPLC)

The HPLC system consisted of a LC-10Ai solvent delivery module (Shimadzu, Kyoto, Japan), SIL-10ADvp automatic injector (Shimadzu), SCL-10Avp system controller (Shimadzu) and a 1046A fluorescence detector (Hewlett-Packard, Palo Alto, CA, USA). Peaks were analysed and quantified with VP-CLASS (version 6.14 SP4).

Results and discussion

Influence of DTT on palmitoyl-CoA stability

The addition of DTT is considered to have a beneficial effect on SPT activity by protecting active site cystein residues from oxidation. However, the presence of DTT might also influence the stability of palmitoyl-CoA due to the reduction of the thioester bond in activated fatty acids. To determine the effect of DTT on substrate stability we incubated palmitoyl-CoA with DTT in concentrations from 0 to 10 mM. The DTT dependent release of free CoA is illustrated in Figure 1. DTT concentration of 5 mM, as it was suggested originally by Merrill and Williams (10, 11) did not significantly influence palmitoyl-CoA concentrations within 10 min of incubation. However, about 50% of the palmitoyl-CoA was reduced after 60 min. This considerable reduction in substrate concentration has to be considered when performing the assay with longer incubation times. Although the presence of DTT might have a beneficial effect, we never observed a reduced SPT activity due to oxidation, even in samples which were stored up to 6 months at -20 °C. Therefore we believe that the presence of DTT in the assay is not essential to maintain SPT activity. However, if DTT is desired, the concentration should be limited to 0.1 - 0.5 mM.

Sample preparation and acyl-CoA thioesterases

SPT activity is usually determined in microsomal preparations. This results in an improved specific activity of the enzyme since cytoplasmatic proteins are largely removed during the microsomal preparation. More important, the use of microsomes also reduces the number of interfering enzymes like

acyl-CoA thioesterases or non-specific hydrolases which compete with the SPT for the substrate palmitoyl-CoA. However, the preparation of microsomes is time consuming, requires an ultracentrifuge and needs rather large amounts of tissue and cells. Furthermore, the resolubilisation of the pelleted microsomal membranes in the absence of a detergent is critical and results frequently in inhomogeneities of the membrane suspensions. The possibility to measure SPT activity directly in cell lysate would significantly simplify these preparative steps. However, the major obstacle for the use of cell lysate is the presence of highly active acyl-CoA thioesterases which results in a fast hydrolysis of the substrate palmitoyl-CoA (11). Acyl-CoA thioesterases are a ubiquitously expressed family of enzymes which show high activities in the cytosol of most eukaryotic cells. Their exact physiological role is not fully clear, but probably important to maintain appropriate intracellular levels of acyl-CoA, free CoA, and free fatty acids (15).

To reduce this perturbing background activity, we aimed to block the thioesterase activity without affecting SPT activity. Thioesterase activity is influenced by several detergents, some lipids and divalent ions like Mn^{2+} and Ni^{2+} (16-19). We investigated several substances in regard to their inhibitory effect on thioesterases and their interference with SPT activity. We found that sucrose monolaurate (SML) showed the best inhibitory effect on thioesterase activity without a detectable inhibitory effect on SPT activity (Table 1). The residual thioesterase activity in cell lysate with 0.1% SML was comparable to the thioesterase activity in microsomes (Figure 2A). SPT activity in microsomes was 50 pmol/min/mg, which is in the range of previously reported results (2.6 to 127 pmol/min/mg in different rat tissues) (11, 20, 21). SPT activity in total cell lysate without any additions was about 6-fold lower. The addition of 0.1% SML significantly improved SPT activity and the activity was comparable to microsomes. In the presence of 0.1% SML the reaction was linear for up to 60 min (Figure 2B). Therefore the assay can be performed over a longer time span which leads to more product and an improved signal-to-noise ratio. The addition of 0.1% SML to the microsomal preparation did not further increase SPT activity.

A comparison of SPT activity in the various preparations of HEK293 cells is summarised in Table 1.

Optimal substrate concentration

The K_m for L-serine was reported to be in the range of 0.1 mM to 1.8 mM for the mammalian SPT (11, 22-24). We re-determined K_m and V_{max} with the new assay conditions (Figure 3). L-serine shows a

regular Michaelis-Menten kinetics while palmitoyl-CoA shows an inhibitory effect at higher substrate concentrations. This substrate inhibition was already reported earlier for the mammalian (11, 25, 26) and for the plant enzyme (24). The maximum of activity was seen at a concentration of approx. 0.05-0.1 mM palmitoyl-CoA (Figure 3B). A K_m of 1.2 mM for L-serine was determined by an Eadie-Hofstee plot. This shows a rather low affinity of SPT towards its substrate L-serine. Commonly the SPT assay is performed at L-serine concentrations between 0.5 and 1 mM. This is far from saturating conditions which are required to measure enzyme activity at its maximal velocity (V_{max}). However, increasing L-serine concentrations in the assay bears the disadvantage that also the amount of radioactive L-serine must be increased in order to maintain the specific radioactivity of the substrate. This is often unfavourable due to cost, health and security reasons. We therefore aimed to establish a non-radioactive, HPLC-based detection method to circumvent the need for radioactivity and to perform the assay under optimal substrate conditions. The used HPLC detection is based on the method of Riley and colleagues (27). Since 3KDS, as the direct product of the SPT reaction, cannot be efficiently detected with this method we converted the 3KDS chemically with sodium borohydride ($NaBH_4$) to sphinganine. This reaction is not stereospecific and results in the formation of erythro- and threo-sphinganine. These two products can be derivatised with ortho-phthalaldehyde (OPA) and quantified by HPLC (Figure 4). Total SPT activity is reflected by the sum of both diastereoisomers. In the negative control, SPT activity was specifically blocked by the addition of myriocin – a commonly used SPT inhibitor (28). Trace amounts of free sphinganine might also be found in the control extract, so that the net SPT activity is calculated by the difference between sample and control.

The detection by HPLC might be biased since the SPT product, 3KDS, could potentially get also acylated by the ceramide synthase (CerS). The diacyl form of 3KDS would be not visible in the HPLC since the primary amine is blocked by the N-linked fatty acid and can not form a fluorescent derivate with OPA. To exclude this possibility, we performed the assay in the presence and absence of fumonisins B₁ (FB1), a specific inhibitor of CerS (29). We found that the presence of FB1 had no influence on the detected sphinganine levels (data not shown).

The limit of detection (LOD) for the radioactivity- and HPLC-based method was compared. The LOD was defined as the activity for which the standard deviation exceeds 20%. Under the chosen condition, the LOD for the HPLC-based detection was 0.1 pmol/min versus 2 pmol/min for the radioactive assay.

This means that the detection limit of the HPLC-based method is about 20 times lower compared to the radioactive method.

However, both methods showed a very close correlation ($r^2 = 0.9519$) (Figure 5B). This demonstrates that the product determination by HPLC is as specific as the radioactive assay and not biased by the presence of an underlying metabolite which might be misinterpreted in the HPLC chromatogram. A further important advantage of the HPLC-based detection is the possibility to add an internal standard to correct for variations in the preparative steps. We used C₂₀-sphingosine or preferably C₁₇-sphingosine, a non-naturally occurring sphingoid base, as an internal standard. Although the HPLC-based method shows significant improvements in terms of sensitivity and variability, it is on the other hand more laborious and time consuming in comparison to the radioactive assay.

Conclusions

In this work we aimed to improve various aspects of the commonly used method to determine SPT activity. One of the major improvements is the possibility to measure activity directly in total cell lysate. We showed that the presence of 0.1% SML significantly inhibits the thioesterase background activity which interferes with the SPT activity measurements. Under these conditions, SPT activity in the total cell lysate was similar to that in microsomes. This adaptation allows it to perform the assay with significantly smaller amounts of tissue and cells. The addition of 0.1% SML also significantly improves the linearity of the reaction. In the presence of 0.1% SML, SPT activity was linear for up to 60 min which resulted in an improved signal-to-noise ratio. We furthermore identified a negative effect of DTT on the stability of palmitoyl-CoA and suggested to reduce or omit the addition of DTT to the assay. Finally, we introduced a non-radioactive, HPLC-based detection method, which makes it possible to measure SPT activity under saturating substrate concentration. This HPLC-based method shows a 20-fold lower detection limit in comparison to the radioactive detection.

We think that these adaptations lead to significant improvements in the robustness, sensitivity and reproducibility of the SPT assay.

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Figure legends

Figure 1: Reduction of palmitoyl-CoA as a side reaction in the presence of different DTT concentrations.

The release of free CoA was monitored via the pyruvate dehydrogenase reaction as described under experimental procedures.

Figure 2: (A) SPT and acyl-CoA thioesterase activities in total lysate and microsomes prepared from HEK293 cells in the presence and absence of 0.1% SML. Low SPT activity and high thioesterase activities were seen in total lysate in the absence of SML. The addition of 0.1% SML increased SPT activity 6-fold and reduced acyl-CoA thioesterase activities by 90%. SPT activity in total lysate in the presence of 0.1% SML was similar to the activity in microsomes. The addition of 0.1% SML to the microsomal preparation did not further improve SPT activity. (B) The SPT reaction in microsomes and total cell lysate + 0.1% SML was linear over 60 min. SPT activity in total cell lysate is reduced by 80% in the absence of SML.

SPT activity was assayed using [^{14}C]-L-serine as described in the appendix. For comparability, the activities were normalised to total amount of extracted protein. $n=3$

Figure 3: Influence of substrate concentration on SPT activity. SPT activity was determined using the HPLC-based assay as described in the appendix. For (A) palmitoyl-CoA concentrations were kept at 50 μM and for (B) L-serine concentrations were kept at 2 mM. Error bars indicate standard deviation. $n=3$

Figure 4: HPLC chromatograms of threo-sphinganine (thr-Sa), erythro-sphinganine (ery-Sa) and C_{20} -sphingosine (C_{20}). (A) Standard solutions: 100 pmol threo- / erythro-sphinganine and 100 pmol C_{20} -sphingosine. (B) Chromatograms of a SPT activity assay with (dashed line) and without (solid line) the addition of NaBH_4 . (C) Overview of the SPT reaction and the following reduction of 3-ketodihydrosphingosine (3KDS) to sphinganine.

Figure 5: Limit of detection (LOD) and correlation. (A) For a maximal assay variance of 20% (dotted line) the HPLC method had a LOD of 0.1 pmol/min, and the [^{14}C]-L-serine based assay a LOD of 2 pmol/min. The HPLC-based detection was therefore about twenty times more sensitive compared to the

radioactive assay. (B) Correlation of the radioactivity- and HPLC-based detection method. Activity assay was performed according to the method described in the appendix followed by the addition of NaBH_4 . The final chloroform phase was split and either analysed on the scintillation counter or by the described HPLC method. Both methods showed a close linear correlation with a correlation coefficient of $r^2=0.9519$.

Table 1: Specific serine palmitoyltransferase activity in various lysate preparations of HEK293 cells. Activities were determined using the radioactivity-based assay. (SML: sucrose monolaurate)

Cell preparation	Thioesterase inhibitor	SPT activity [pmol/min/mg total protein] ^a	
Total cell lysate	none	2.4 ± 0.3 ^b	2.4 ± 0.3 ^c
	0.1% SML	12.4 ± 1.6 ^b	12.4 ± 1.6 ^c
Microsomes	none	14.0 ± 2.8 ^b	50.3 ± 10.2 ^c
	0.1% SML	12.9 ± 1.2 ^b	46.5 ± 4.4 ^c
Supernatant (100'000g)	none	not assayed	not assayed
	0.1% SML	0.3 ± 0.4 ^b	0.5 ± 0.6 ^c

^a Mean ± standard deviation, *n*=3

^b SPT activity normalised to the total amount of extracted protein

^c SPT activity normalised to protein amount of the assayed fraction

Figure 1

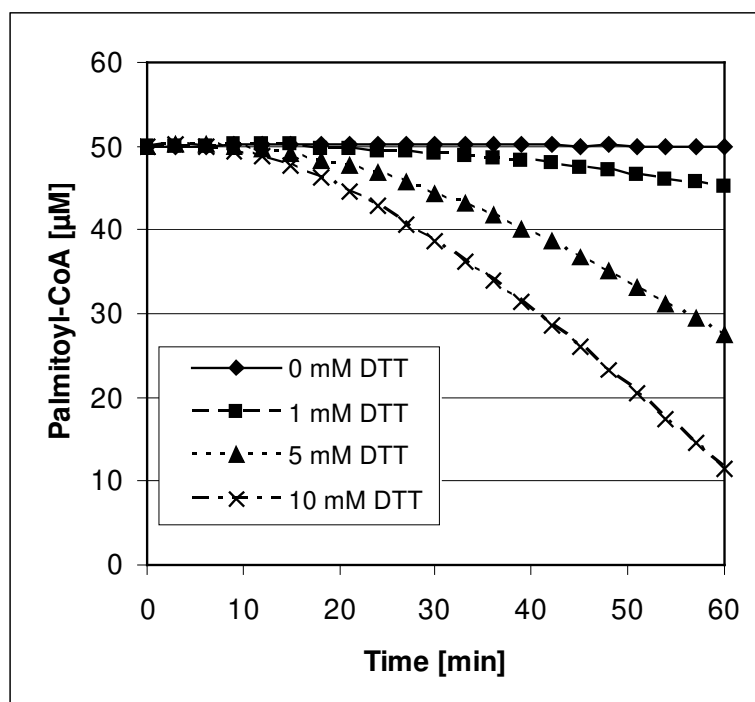


Figure 2

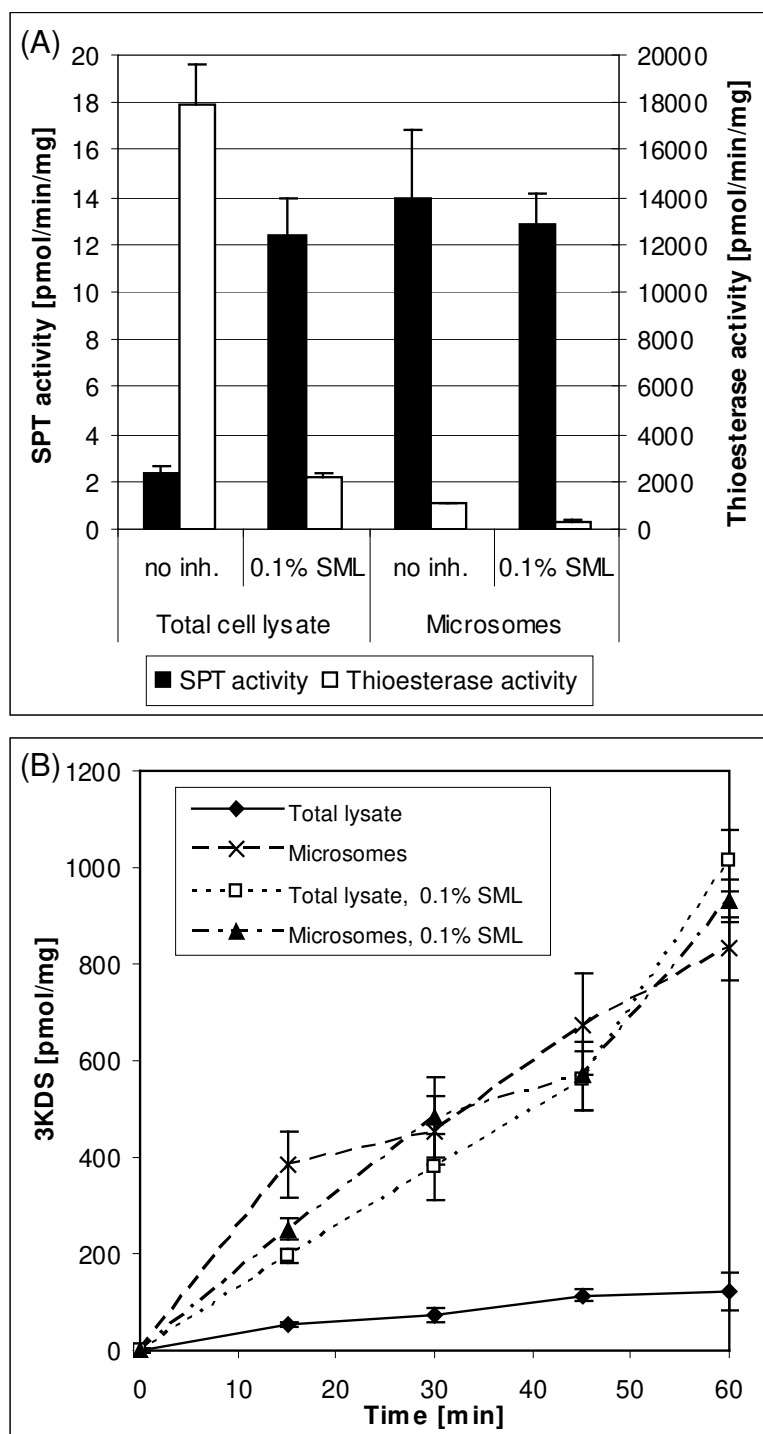


Figure 3

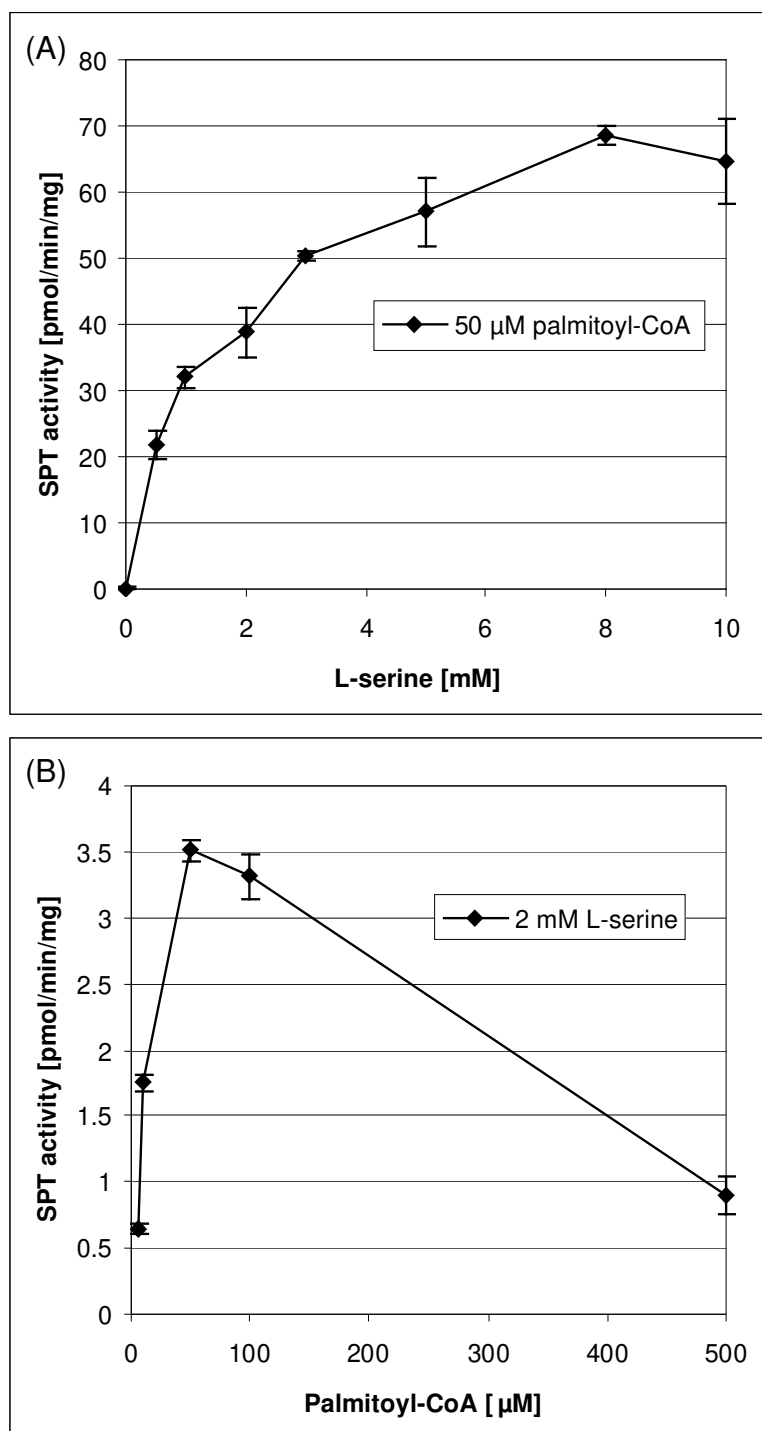
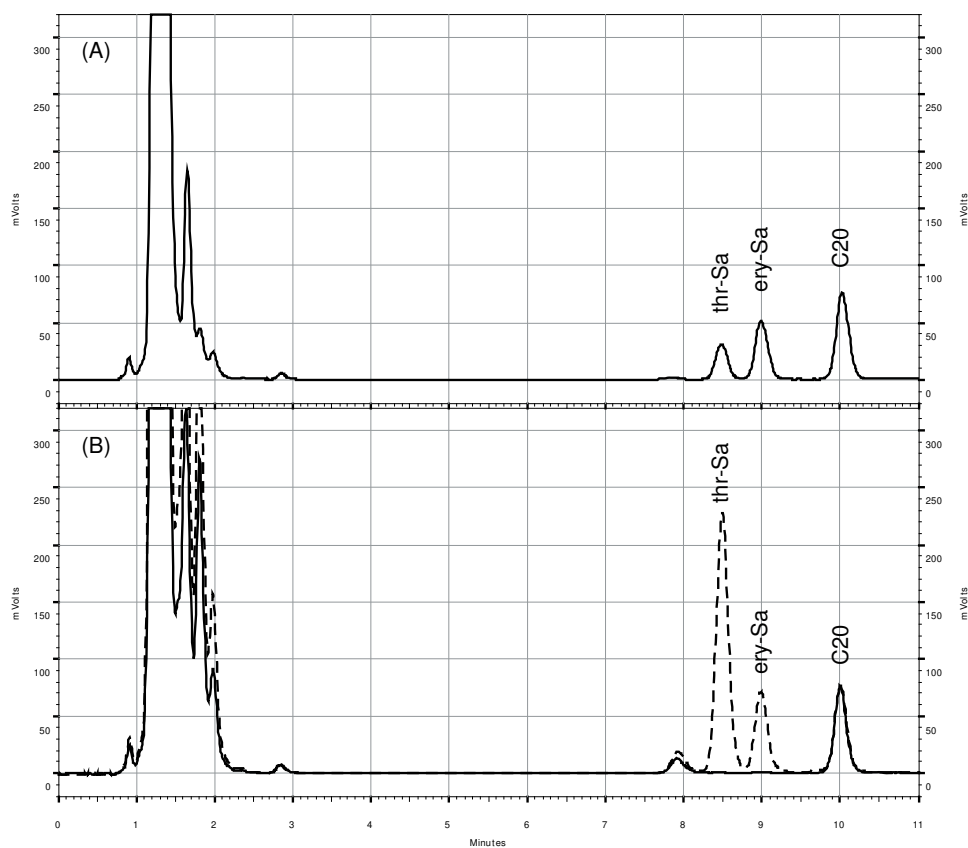


Figure 4



(C)

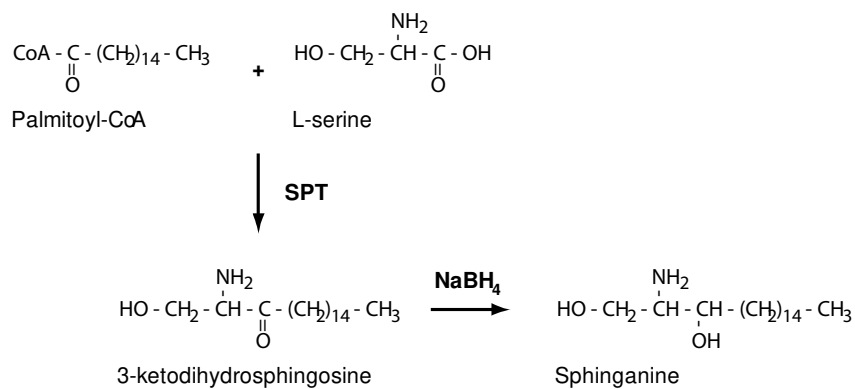
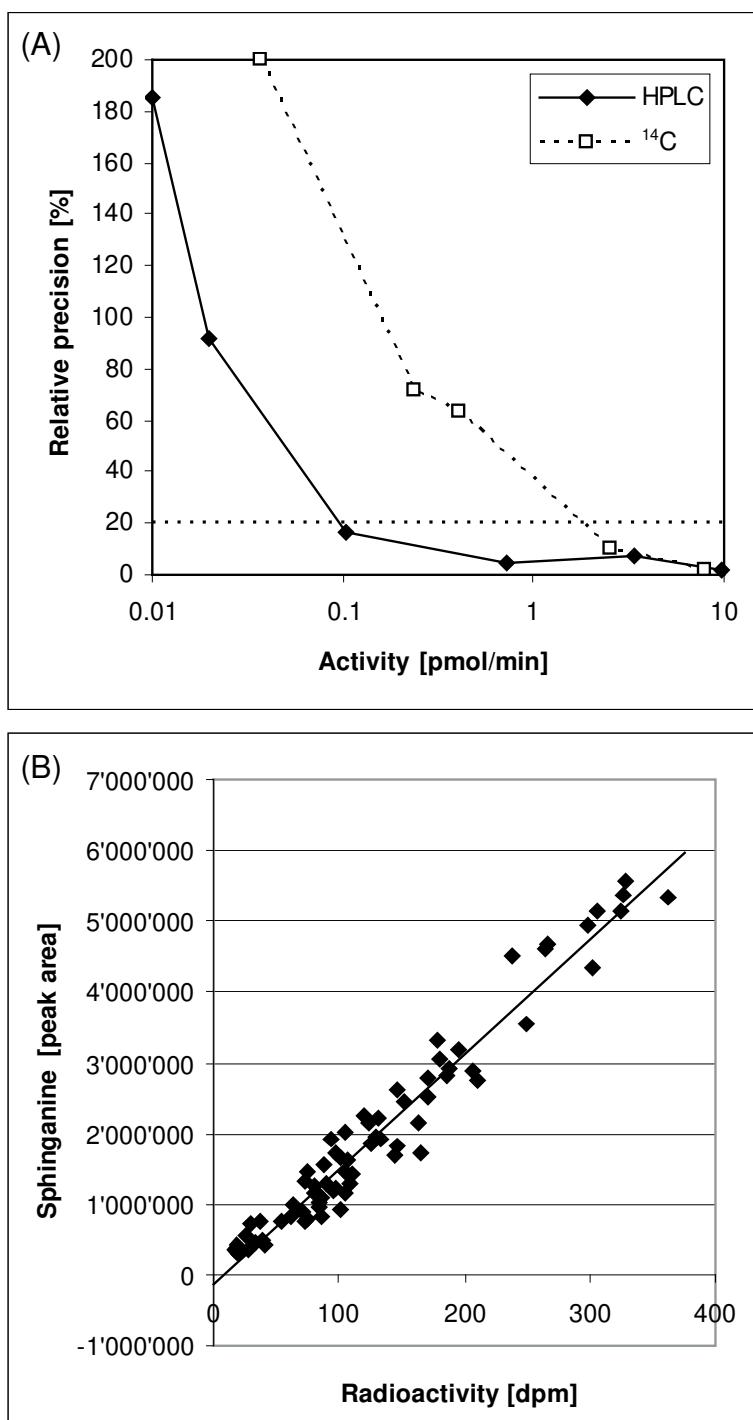


Figure 5



Appendix: Sample protocol for an improved serine palmitoyltransferase activity assay

This protocol describes an improved assay to determine serine palmitoyltransferase activity. Two different detection methods are suggested:

- Radioactivity-based assay: [^{14}C]-labelled L-serine is incorporated into 3-ketodihydrosphingosine, which is quantified by liquid scintillation counting.
- HPLC-based assay: 3-ketodihydrosphingosine is chemically reduced by NaBH_4 to threo- and erythro-sphinganine, whose ortho-phthalaldehyde derivatives are separated by HPLC and quantified by fluorescence detection.

Reaction conditions:

- Reaction buffer: 50 mM HEPES (pH 8) containing 1 mM EDTA and 0.1% (wt/vol) sucrose monolaurate
- 5 mM L-serine (HPLC-based assay) or 0.5 mM L-serine (radioactivity based assay)
- 50 μM palmitoyl-CoA
- 20 μM pyridoxal 5'-phosphate

1. General Reagents

- a. Stock solutions: L-serine (200 mM), palmitoyl-CoA (5 mM), pyridoxal 5'-phosphate (5 mM), HEPES-NaOH (0.5 M, pH 8), EDTA (0.5 M, pH 8), sucrose monolaurate (10% (wt/vol)), myriocin solution (1 mg/ml in methanol), NH_4OH (2 N)
- b. Alkaline water: 0.1 ml NH_4OH (2 N) in 100 ml of water
- c. Methanolic KOH: Dissolve 0.7g KOH (platelets) in 100 ml of methanol.
- d. Methanol/KOH: CHCl_3 (4:1): Mix 4 vol of methanolic KOH with 1 vol of CHCl_3 ; prepare fresh immediately before use.
- e. Lysis buffer: Mix 1 ml of HEPES-NaOH (0.5 M, pH 8), 20 μl of EDTA (0.5 M, pH 8) and 100 μl of sucrose monolaurate (10% (wt/vol)). Bring to a volume of 10 ml with water.

The following reagents are only required for the radioactivity-based assay:

- f. L-[U-¹⁴C]-serine (50 µCi/ml)
- g. 20x [¹⁴C] assay mix (incl. [¹⁴C]-L-serine): example for 250 µl (suitable for 25 samples):
Mix 12.5 µl of L-serine (200 mM), 20 µl of pyridoxal 5'-phosphate (5 mM), 50 µl of palmitoyl-CoA (5 mM), 100 µl of L-[U-¹⁴C]-serine (50 µCi/ml) and 68 µl of water.
- h. Liquid scintillation cocktail (e.g. Ultima Gold, PerkinElmer)

The following reagents are only required for the HPLC-based assay:

- i. 20x HPLC assay mix: example for 250 µl (suitable for 25 samples):
Mix 125 µl of L-serine (200 mM), 20 µl of pyridoxal 5'-phosphate (5 mM), 50 µl of palmitoyl-CoA (5 mM), and 55 µl of water.
- j. NaBH₄ (5 mg/ml in water): Prepare immediately before use.
- k. Sphingolipid standard solutions: C₁₇-sphingosine (1 mM in ethanol), threo- / erythro-sphinganine (1 mM in ethanol)
- l. HPLC standard mix: Mix 5 µl of C₁₇-sphingosine (1 mM in ethanol) and 5 µl of threo- / erythro-sphinganine (1 mM in ethanol). Bring to a volume of 7.5 ml with methanol:ethanol:water (85:47.5:17.5).
- m. Methanol:CHCl₃:0.1 M KOH + C₁₇-Sphingosine (internal standard): Add 0.2 µl of C₁₇-sphingosine (1 mM in ethanol) per ml methanol/KOH:CHCl₃. 0.5 ml is needed per sample.
Prepare always fresh immediately before use.
- n. Boric acid (3% (wt/vol), pH 10.5): Adjust pH with KOH as necessary. Check pH before use.
- o. Ortho-phthalaldehyde (OPA, 50 mg/ml in ethanol)
- p. OPA reagent: Mix 10 µl OPA (50 mg/ml in ethanol), 990 µl boric (3% (wt/vol), pH 10.5)) and 0.5 µl of 2-mercaptoethanol. Store in dark, 4 °C. Prepare fresh daily.
- q. Potassium phosphate buffer (5mM, pH 7): Mix 5 mM K₂HPO₄ (0.87 g in 1000 ml of water) and 5 mM KH₂PO₄ (0.68 g in 1000 ml of water) in a ratio of 61:39 (vol/vol) and adjust pH as necessary.
- r. Running buffer for HPLC: methanol:5 mM potassium phosphate buffer (pH 7) (90:10, vol/vol)

2. Preparation of total cell lysate

All steps at 4 °C

- a. Cell monolayer in a 10-cm dish (80 - 90% confluent)
- b. After washing twice with PBS, suspend cells in 1 ml of lysis buffer.
- c. Sonicate for 15 s at 50% power and 50% pulsation. Solution should be homogeneous. No clumps should be visible.
- d. Centrifuge at 2'500g for 2 min to remove debris.
- e. Take supernatant and determine protein concentration. Total protein concentration should be around 2 mg/ml. Dilute with lysis buffer if desired.

3. SPT activity assay

Do the reaction in a 2 ml polypropylene reaction tube.

- a. Take 190 µl of total cell lysate (about 200 - 400 µg total protein) and 10 µl of the corresponding 20x assay mix ($[^{14}\text{C}]$ or HPLC). Vortex briefly and keep samples on ice. For negative control, add 2 µl of myriocin solution. To correct for a methanol effect also add 2 µl of methanol to the samples.
- b. To start the reaction transfer tubes to a water bath. Incubate at 37 °C for 60 min.

The following step is only necessary for the HPLC-based assay:

- c. Add 50 µl of NaBH_4 (5 mg/ml) and let react for 5 min at room temperature.

4. Lipid extraction and product detection

- a. Add 0.5 ml of methanol/KOH: CHCl_3 (for the radioactivity-based assay) or 0.5 ml of methanol: CHCl_3 :0.1 M KOH + C_{17} -sphingosine (for the HPLC-based assay) and mix intensively (vortex).
- b. Add 0.5 ml CHCl_3 .
- c. Add 0.5 ml alkaline water and 100 µl of 2N NH_4OH , mix intensively (vortex).
- d. Centrifuge (12'000g, 1 min at room temperature).
- e. Remove the upper phase with gentle aspiration.

- f. Wash the lower phase twice with 900 µl of alkaline water.

The following steps are only necessary for the radioactivity-based assay:

- g. Transfer 400 µl of the lower phase to a polyethylene scintillation vial.
- h. Let the CHCl₃ evaporate under a stream of nitrogen.
- i. Add 5 ml of liquid scintillation cocktail and count the incorporated radioactivity by a scintillation counter. For activity calculations take into account that the transferred 400 µl CHCl₃ are 2/3 of the total CHCl₃ phase (600 µl).

The following steps are only necessary for the HPLC-based assay:

- j. Transfer 400 µl of the lower phase to a new 2 ml polypropylene vial.
- k. Let the CHCl₃ evaporate under a stream of nitrogen.
- l. Re-dissolve the residue in 150 µl of methanol:ethanol:H₂O (85:47.5:17.5).
- m. In an additional vial, add 150 µl of HPLC standard mix. This vial will provide the defined standard peaks.
- n. Add 5 µl of OPA reagent to each vial and let react for 2 h in the dark.
- o. Analyse samples by HPLC using the following conditions:
- C18 reverse-phase column (100 Å pore size, 3 µm particle size, 4×125 mm column)
 - Injection volume: 50 µl
 - Flow rate: 1.0 ml/min
 - Pump program:
 - 0 - 20 min: methanol:5 mM potassium phosphate buffer (pH 7) (90:10, vol/vol), isocratic
 - 20 - 25 min: methanol (100%), isocratic (wash)
 - 25 - 30 min: methanol:5 mM potassium phosphate buffer (pH 7) (90:10, vol/vol), isocratic
 - Measure OPA fluorescence at an excitation wavelength of 335 nm and an emission wavelength of 440 nm.
- p. Retention times for C₁₇-sphingosine, threo- and erythro-sphinganine standards are approximately 5.5, 8.5 and 9 min.

- q. SPT activity is calculated using the following formula:

$$v = ((thr_{sample} + ery_{sample}) \times C17_{std} \times 100 \text{ pmol}) / ((thr_{std} + ery_{std}) \times C17_{sample} \times p_{tot} \times t)$$

Where v is the specific SPT activity in pmol/min/mg, thr and ery are the areas of threo- and erythro-sphinganine peaks, $C17$ is the area of C_{17} -sphingosine peak, p_{tot} is the total amount of protein in mg and t is the reaction time in minutes. The subscripts $_{sample}$ and $_{std}$ stand for peaks from the samples and from the HPLC standard mix, respectively.